Relevance of Platelet-independent Effects of Aspirin to Its Salutory Effect in Atherosclerosis-related Events

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There is a close inter-relationship between oxidative stress, coagulation, inflammation, and smooth muscle cell growth as key components of atherosclerosis (Fig. 1). As an analgesic and anti-pyretic, aspirin has been in use for over a century. It acetylates the COX enzyme, irreversibly inhibiting the formation of prostaglandin. Its action on platelet TxA₂ has highlighted its role as an anti-thrombotic agent in cardiovascular patients. Over the last two decades, unique anti-inflammatory properties of aspirin not shared by other non-steroidals have been discovered. Aspirin biotransforms into salicylate, which has diverse but potent anti-inflammatory properties. As we strive to better understand the concepts of atherogenesis, chronic inflammation, oxidative stress, and endothelial activation, these novel effects of aspirin provide new insights as to how this wonder drug works. These effects of aspirin alter many, if not all, components of the atherogenesis cascade shown in Fig. 1. *J Atheroscler Thromb*, 2005; 12: 185–190.

Key words: Adesion molecules, Inflammation, oxidative stress, smooth muscle cell growth

Introduction

In this era of ever-increasing poly-pharmaceutical approaches to cardiovascular diseases, aspirin has come to be the leading drug in the reduction of cardiovascular mortality, followed by beta-blockers, statins and ACE-inhibitors. Aspirin's widespread use as a primary and secondary preventive agent for cardiovascular disease has been well established among physicians. It reduces the evolution of vascular events by 20–25%, and commands a class I indication in the treatment of acute coronary syndrome by the American Heart Association/American College of Cardiology Task Force (1, 2).

The medicinal benefits of aspirin have been known to mankind for over a century, long before it's mechanism of action was understood (3). Salicin was in therapeutic use in the form of white willow bark extract for almost

2000 years. Acetylsalicylic acid was first synthesized in the 1850s for use in treating rheumatism as an anti-inflammatory, analgesic and anti-pyretic agent. It was in 1971 that Sir John Vane demonstrated that the inhibition of prostaglandin synthesis was the mechanism of action of aspirin. It is now well accepted that the beneficial effects of aspirin in the secondary prevention of myocardial infarction are primarily mediated by an irreversible inhibition of the cyclooxygenase-1 (COX-1) enzyme, resulting in the inhibition of thromboxane A₂ (TxA₂) formation during the life span of platelets which are unable to regenerate COX-1.

Aspirin has found a unique place in the treatment of atherosclerosis and its manifestations which result from an interaction between platelet activation, endothelial inflammation/activation, and oxidative stress (Fig. 2). The usual risk factors for coronary atherosclerosis, which include diabetes mellitus, hypertension, dyslipidemia and smoking, are associated with intense oxidative stress to intense inflammation. It is critical to understand the process of atherogenesis and events that lead to plaque rupture to understand the benefits of aspirin therapy. We

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Fig. 1. Inter-relationship between oxidative stress, inflammation, coagulation and smooth muscle cell growth.

review here some of the work that shows aspirin to be a potent vasoprotective agent through its well known platelet-dependent and the emerging platelet-independent mechanisms.

Platelet-dependent Effects of Aspirin

The direct anti-thrombotic effect of aspirin is believed to be mediated by inhibition of the COX-1 enzyme in platelets (4). This effect is mediated by the irreversible acetylation of COX-1 at a serine residue near the catalytic site, permanently altering the site for arachidonic acid resulting in a decreased production of TxA_2 , a potent vasoconstrictor and platelet pro-aggregant (5). This irreversible inhibition of COX-1 provides a sufficient explanation for the selective inhibition of COX by low-dose aspirin in platelets that, in contrast to vascular endothelial cells, cannot synthesize COX *de novo*.

This anti-platelet effect presumably translates into significant clinical benefits in patients with unstable angina, acute myocardial infarction, coronary artery bypass graft and percutaneous coronary interventions. In all these conditions, there is evidence for enhanced platelet aggregation. Although most of the benefits of aspirin in vascular disease have been attributed to inhibition of platelet TxA_2 synthesis, it should be noted that direct TxA_2 receptor antagonists have not been found to be of any clinical benefit (6). Further, other agents that inhibit the COX enzyme such as indomethacin or ibuprofen do not exert the same cardioprotective effects as aspirin (6). These observations raise concern regarding the major mechanisms by which aspirin exerts its salutary effect.

Platelet-independent Effects of Aspirin

Effect on oxidative stress

There is compelling evidence that an excess of oxidative stress beyond the body's ability to cope contributes significantly to endothelial damage during atherogenesis. Oxidant burden seems to correlate with cardiovascular

risk factors (7, 8). Oxidative stress is a result of the release of reactive oxygen species (ROS) and other nitrosylated products. ROS, in particular, mediates atherogenic signals in vascular cells. Growth factors such as angiotensin II also stimulate production of ROS (8) which activate several intracellular responses, including the activation of NF- κ B with the subsequent upregulation of adhesion molecules (9, 10). A number of studies show that aspirin, but not indomethacin, may exert an inhibitory effect on the generation of ROS and associated intercellular signaling pathways (11, 12).

The atherosclerotic arteries also exhibit the deposition of oxidatively modified low density lipoproteins (ox-LDL) (13) which along with shear stress, endothelin and angiotensin II, upregulate the activation of ox-LDL-specific receptors, such as LOX-1, leading to endothelial activation, dysfunction, apoptosis, and MMP-1 synthesis as well as platelet activation (14).

We have observed that aspirin, in a dose- and time-dependent fashion, reduced ox-LDL-mediated LOX-1 expression, MMP-1 expression and activity, p38MAPK activation and superoxide anion generation in human coronary artery endothelial cells (15). Interestingly, we have observed that the treatment of human coronary artery endothelial cells with salicylate, but not indomethacin, resulted in effects similar to those of aspirin (15).

In earlier studies, Chen *et al.* (16) demonstrated in our laboratory that ox-LDL caused a concentration-dependent increase in thrombin-induced platelet aggregation and decreased activity. These effects were reversed by the pretreatment of platelets with L-arginine, the precursor (Fig. 3). O'Kane *et al.* (17) showed that basal NOS activity in human platelets was increased significantly by intravenous aspirin. In the same study, incubation with aspirin, but not with indomethacin or ibuprofen, increased NOS activity in platelets, suggesting that aspirin acts through a mechanism independent of COX inhibition. It is well known that oxidative stress causes the breakdown of NO.

Grosser and Schröder (18) showed that preincubation with aspirin, but not salicylate or indomethacin, protected endothelial cells from hydrogen peroxide-mediated toxicity and increased their viability in a concentration-dependent fashion. This effect was abrogated in the presence of a NO scavenger and L-arginine analogs. They also showed that aspirin enhanced activity and intracellular cyclic GMP accumulation in endothelial cells (18). Similar protection of endothelial cells from oxidative stress by vitamin E had been shown earlier (19). Aspirin has also been shown to prevent hydrogen peroxide-induced caspase-3, caspase-9 and NF- κ B activation in a dose-dependent manner through inhibition of phosphorylation and degradation of I κ B α and I κ B β (20). Aspirin may well be more potent in endothelial protection than vitamin E (19).

Atherosclerosis: Role of Platelets, Endothelial activation and Leukocytes

Fig. 2. Model describing early pathophysiological processes in atherosclerosis involving platelet activation, endothelial injury and dysfunction, oxidative stress and inflammation. Abbreviations: ROS-reactive oxygen species.

In recent studies, the induction of ferritin has been shown to provide marked cellular protection by rapidly sequestering free cytosolic iron, the crucial catalyst of oxygen-centered radical formation via the Fenton reaction in biological systems (20, 21). Cells overexpressing ferritin protein are more resistant to oxidative injury (22). Thus, ferritin, which was thought to function merely as a “housekeeper” iron storage protein, has emerged as a critical and fast-acting endogenous cytoprotectant that plays an important role in cellular antioxidant-related defense mechanisms (23, 24). In cultured endothelial cells, aspirin has been shown to increase the synthesis of ferritin, an effect not shown by other nonsteroidal anti-inflammatory drugs, indomethacin or diclofenac (25). Thus, it seems plausible that by increasing the synthesis of iron-scavenging ferritin, aspirin may specifically withdraw iron ions from the site of oxygen radical formation, and may thus effectively interrupt the reaction cascade leading to oxidative stress and tissue damage.

The transcription factor NF- κ B is a ubiquitous effector of the inflammatory response, and promotes the expression of genes encoding cytokines, cytokine receptors, chemoattractant proteins, and the adhesion molecules VCAM-1, ICAM-1, and E-selectin (9, 10). NF- κ B cognate regulatory elements are present in the promoters of these three genes and are essential for their transcriptional induction by the inflammatory cytokines TNF- α and IL-1. Costanzo *et al.* (11) demonstrated that in cultured hu-

man umbilical vein endothelial cells, aspirin can abolish the angiotensin II-mediated induction of NF- κ B transcriptional activation. In contrast, exposure to indomethacin did not produce any significant inhibition of NF- κ B activity, suggesting that these two COX inhibitors target distinct signaling pathways in this system.

Effect on endothelial activation/dysfunction

While increased platelet activity in fact plays a crucial role during acute thrombosis, it is the injured and dysfunctional endothelium that facilitates the adhesion of platelets and other blood cells to the vascular wall and can thus be considered a primary factor in atherogenesis and the formation of a thrombus (26). Pro-inflammatory cytokines released by the vessel wall components and circulating cells are considered critical to the initiation and progression of atherosclerosis. Consistent with this concept, plasma levels of macrophage colony stimulating factor (MCSF), IL-1 β , IL-6, and CRP are significantly elevated in patients with coronary atherosclerosis, in one study MCSF, IL-6, and CRP were all reduced after 6 weeks of aspirin therapy (27). This phenomenon may in part explain aspirin’s therapeutic action.

As mentioned earlier, aspirin may reduce the direct toxic effect of pro-oxidative stimuli and enhance the viability of endothelial cells (18). This may be mediated by enhancement of NO synthase activity and subsequent intracellular cyclic GMP accumulation. In keeping with this

Fig. 3. Representative experiments showing stimulation of platelet aggregation when platelets were incubated with different concentrations of ox-LDL. The stimulation of platelet aggregation with 1 to 2.5 U/mL thrombin was attenuated by prior incubation of platelets with L-arginine

concept, the impaired forearm vasodilatation in hypercholesterolemic patients in response to acetylcholine was partially corrected when patients were treated with aspirin, suggesting another therapeutic action of aspirin (28). In another study hypertensive patients, a similar effect of improvement in flow-mediated dilatation and increased cGMP production was observed after treatment with aspirin (29).

Acute myocardial ischemia is associated with endothelial activation as evident from enhanced platelet adhesiveness at the site of ruptured plaques. In our laboratory, Ranganathan *et al.* (30) showed that aspirin was able to enhance p53 expression in human coronary artery endothelial cells exposed to ox-LDL. In addition to platelet inhibition, protection and preservation of the endothelial activity be an important target for the tissue-protective effects of aspirin.

Effect on leukocyte adhesion and chemotaxis

Increased white blood cell adhesion to the activated endothelium leads to leukocyte accumulation and transmigration to the subendothelial layers, which contribute to the progression of atherosclerotic plaques. Leukocyte adhesion is characterized by the expression of a number of molecules such as E-selectin, ICAM-1 and VCAM-1, which support their adhesion to the endothelial cells via interaction with counterligands VLA-4 (CD29/49d), LFA-1 (CD11a/CD18), and Mac-1 (CD11b/CD18), all of which are expressed in human atherosclerotic plaques in large numbers (31, 32).

Voisard *et al.* (33) have shown that aspirin decreases the adherence of monocytes or CD4 (+) lymphocytes to

human coronary artery endothelial cells. These investigators also showed that aspirin inhibits the proliferation of cultured human coronary artery smooth muscle cells. Weber *et al.* (34) also showed that aspirin in a dose-dependent fashion inhibits TNF- α induced NF- κ B mobilization, VCAM-1 and E surface expression. Adhesion of U937 monocytes to TNF- α -stimulated human venous endothelial cells was markedly reduced by aspirin. These effects of aspirin were not related to the inhibition of COX activity.

Effect on cell proliferation

Reactive coronary artery smooth muscle cell proliferation is a key event in the development of atherosclerosis and restenosis. As mentioned above, treatment with aspirin caused a significant decrease in the reactive proliferative response of human coronary media smooth muscle cells after the leukocyte attack. In a study in dogs fed a cholesterol-enriched diet for 8 months, aspirin treatment attenuated platelet function, prolonged bleeding time, and prevented abrupt thrombotic occlusion, but, importantly, inhibited neointimal proliferation (35).

Aspirin and sodium salicylate similarly inhibit vascular smooth muscle cell proliferation by arresting the cell cycle in the G₁-S phase (36). Work from our laboratory has shown that aspirin decreases DNA and protein synthesis in endothelial cells by upregulating P53 expression (30). Bellosillo *et al.* (37) also showed that aspirin and salicylate induce apoptosis of chronic lymphocytic leukemia cells through the activation of caspase-3 by COX-independent mechanisms.

These observations indicate that aspirin can decrease endothelial and smooth muscle cell proliferation through cell cycle arrest and other mechanisms. These mechanisms may contribute to the overall effects of aspirin on atherosclerosis-related diseases.

Salicylate-specific actions of aspirin

After the oral administration of an analgesic dose of aspirin, 50% is de-acetylated to salicylate immediately after absorption. The plasma half-life of aspirin is about 15 minutes whereas that of salicylate is between 2 and 30 hours depending upon the dose. Since aspirin is rapidly de-acetylated into salicylate, it is postulated that the anti-inflammatory effects of aspirin are due to the salicylate moiety.

Sodium Salicylate, unlike aspirin, is almost inactive as a direct inhibitor of COX-1 in the vascular wall (38) and does not prevent the formation of TxA₂ in platelet-rich plasma. Instead, it is only a weak inhibitor of TxA₂ in clotting whole blood (39). It is plausible that the anti-inflammatory effects of sodium salicylate are not dependent on the inhibition of prostaglandin synthesis.

High concentrations of salicylates have been shown to inhibit kinases including the MAPK cascade, with the

exception of a few reports (40). NF- κ B is regarded as a key element in the response of cells to inflammatory stimuli. Inhibition of the NF- κ B pathway by aspirin and salicylate was first demonstrated by Kopp and Ghosh (41) followed by several others. Sodium salicylate also serves as a chemical trap for hydroxyl radicals, and has been shown to ameliorate hypoxia/reoxygenation injury in several tissues (42). It has also been suggested that the uncoupling of oxidative phosphorylation by salicylates decreases intra-cellular ATP formation, consequently inducing the release of adenosine into the extracellular fluids in sufficient quantity to exert anti-inflammatory effects (43).

We have observed that, like aspirin, salicylate has inhibitory effects on ox-LDL-mediated LOX-1 expression, MMP-1 expression and activity, p38MAPK and superoxide anion generation in human coronary artery endothelial cells (15). These results suggest that the novel effects of aspirin mediated by its salicylate moiety complement the role of the acetyl moiety in terms of its platelet inhibitory effect.

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